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ASPECTS OF MOLTING AND CALCIFICATION IN THE OSTRACOD *HETEROCYPRIS*

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The ostracod is a marine or freshwater crustacean with a bivalved carapace that encloses the rest of the body. The valves composing the carapace are heavily calcified and, being an integral part of the exoskeleton, they are shed during each molt. The valves of the new exoskeleton are then calcified while the rest of the exoskeleton remains unmineralized.

The calcification process associated with molting in ostracods is not well known. X-ray diffraction studies by Kesling (1951) on *Cypridopsis vidua* determined that the calcium is deposited within the ostracod valve as calcite. Fassbinder (1912), experimenting with the same species, concluded that the immediate source of the calcium used in calcification was from the body of the ostracod rather than from the aqueous environment. This was determined by growing the ostracods in calcium-free water and feeding them calcium-free food, and finding that they could still calcify the new valves after a molt. Van Morkhoven (1962) reported that the animal's diet will affect the calcification of the valves, with rich calcium diets producing secondary thickening and numerous protuberances. Finally, Kesling (1951) proposed that *C. vidua* has no special cells which secrete the shell material; it is formed rather by all the epidermal cells within the duplicature. He believed the ostracod calcification process was different from that of other crustaceans that have been studied.

The objective of this study was to gain some information about this calcification process. Experiments using Ca^{45} as a tracer were conducted to determine whether any calcium is reabsorbed from the valves prior to molting and whether calcium is stored for use in later valve calcification. Autoradiographs were prepared to determine which cells were active in calcifying the new valves.

Materials and Methods

Cultures of *Heterocypris* were started from individuals collected at ponds in Cherry Hills Village, Colorado. These cultures were maintained in "Chlorofree" (Lambert-Kay Inc., Los Angeles) treated tap water at a pH of 7.4. Parboiled rice grains and hay were added as food. Isolated individuals were maintained in small culture dishes containing 22 ml treated tap water at pH 7.4, a one-quarter-inch piece of hay, one-quarter of a rice grain, one drop of detritus from the stock cultures, and some ground calcite to provide a rough substrate. Cultures were maintained at approximately 23° C.

In all the tracer studies the dosage of Ca^{45} (Nuclear Chicago) was one microcurie of the isotope per twenty-two ml of treated tap water. Determination of radioactivity was made by counting the organisms in metal planchets using a

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Nuclear Chicago model 186 decade scaler and a Geiger-Muller tube with a window thickness of 1.4 mg/cm^2.

Cytological and autoradiographic material was prepared by fixing in 5% formalin adjusted to pH 8.0 with NaOH, dehydrating with graded ethyl alcohols, and embedding in Epon 812. The embedded organisms were decalcified by nicking the anterior end of the Epon block to expose the valves, placing the blocks in 1 *N* HCl for one hour, and re-embedding in Epon 812 in a vacuum oven. This technique protected the soft tissues from acid degradation and prevented the loss of cellular calcium.

The material was sectioned at two microns on a Sorvall Mt-1 ultramicrotome. Sections were collected on distilled water at pH 8.0 and mounted on glass slides without any subbing. Sections were stained with either standard hematoxylin 5 minutes at 60° C and Saffranin-0 0.1% for 3 minutes at 60° C, or fast green FCF 0.1%, pH 2.0 at 60° C for five minutes, or toluidine blue (Trump, Smuckler and Benditt, 1961) modified by staining in Columbia staining jars for thirty minutes at 60° C.

Sections of specimens for autoradiography were fixed, embedded, and sectioned as above. Two-micron sections were mounted on slides, dipped in Kodak NTB-2 Nuclear Track Emulsion, and exposed for fourteen days in light-tight boxes. After development they were stained with toluidine blue, dehydrated, and mounted under coverslips.

The mineralogical composition of the valves was determined by X-ray diffraction of powdered valves mounted in a Debye-Scherrer powder camera.

Experimental Procedures

Determination of instar times

Three groups of fifteen animals each were isolated and observed hourly from the time of hatching until the adult stage was reached. The occurrence of each molt and the general condition of each animal was recorded.

Determination of calcium reabsorption

Twenty cultures were established, each containing 22 ml treated tap water and one microcurie Ca^{45}. Ten cultures contained ten animals each; ten cultures contained one animal each. The animals were allowed to molt in the radioactive media. After molting and calcification of the new valves, the animals were rinsed three times in treated tap water and transferred to identical cultures without Ca^{45}. The animals were then allowed to molt in the non-radioactive media, and the discarded valves and the animals were collected, washed, and dried for radioactivity assay.

Controls were prepared by placing ten dead ostracods killed by drying in an identical culture of Ca^{45} for the duration of the experiment. Additional controls were prepared by collecting the discarded non-radioactive valves from the first molt and exposing them to cultures containing Ca^{45} for the time period of the experiment.

Determination of calcium storage prior to molt

Thirty animals in the seventh instar were isolated in individual cultures containing one microcurie of Ca^{45}. The animals were allowed to undergo one com-

plete molting cycle in this medium. At this stage of development the next molt normally occurs at 72 hours; therefore ten animals were removed 24 hours after molting in Ca^{45}, ten after 48 hours, and ten after 72 hours. Each group of organisms was dried on metal planchets after rinsing them three times in treated tap water.

Controls were established by exposing dead animals to the same concentration of Ca^{45} for 24, 48, and 72 hour intervals.

Observations and Results

As observed in our cultures, an ostracod beginning to molt can be distinguished by the development of clear areas along the ventral margins of the valves where they are starting to separate. During this time there is also a slight ballooning of

Table I

Instar time periods under laboratory culture conditions. Molting occurs every one to three days depending on the stage of development attained by the individual

Instar	No. of animals observed	Time in hours between molts		
		Shortest	Longest	Mean
1st to 2nd	45	24	24	24
2nd to 3rd	45	24	48	26
3rd to 4th	45	24	48	29
4th to 5th	39	24	48	43
5th to 6th	36	24	72	53
6th to 7th	34	48	72	62
7th to 8th	34	48	96	74
8th to 9th	31	72	130	77

the animal that causes it to lose its oval shape and to appear more rounded. As this swelling increases, swimming movements become erratic and the edges of the valves are rubbed against the bottom. This activity is interrupted by frequent periods of motionlessness.

The actual discarding of the exoskeleton begins with the scraping of the inner surface of the valves by the thoracic legs followed by the withdrawal of the two pairs of antennae from the old exoskeleton and then the withdrawal of the remaining appendages. Once all the appendages are free, there is a further swelling of the ostracod that causes the old exoskeleton to separate completely from the body. The newly emerged individual appears soft and swollen. The transparent, uncalcified valves are gradually calcified over a period of several hours becoming opaque as calcification is completed.

Determination of instar times

Under laboratory culture conditions molting occurs every one to three days depending on the stage of development of the individual. As may be seen in Table I the younger instars molt more rapidly than the more mature animals and there is considerable individual variation in instar duration.

Determination of calcium reabsorption

Ostracods placed in water containing Ca^{45} do not incorporate Ca^{45} into the valves until the next molt and the calcification of the new valves. If the ostracods are then transferred to non-radioactive water, during the following molt the radioactive valves are shed, and none of the Ca^{45} in them is reabsorbed for use in calcifying the new valves. All the animals (110 individuals) that molted in Ca^{45}-containing water incorporated Ca^{45} into the valves as was shown by radioactive assay of the valves after they had been discarded during the following molt. The paired valves registered approximately 400 to 500 cpm. The ostracods and their new valves (108 individuals), when assayed by drying the whole animal on a

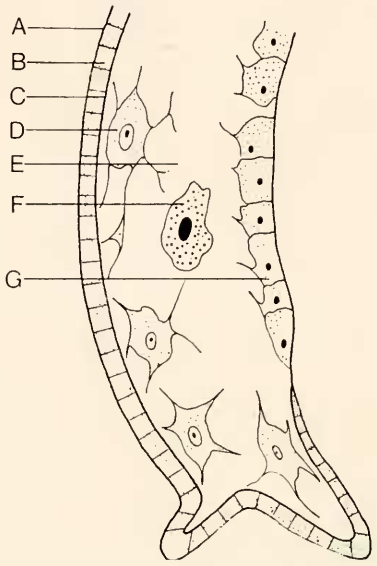

FIGURE 1. A simplified diagram of a section through the ventral margin of the duplicature: A, Outer chitinous layer; B, Calcified valve; C, Inner chitinous layer; D, Cell of outer lamella; E, Interlamellar space; F, Sub-dermal cell; G, Cell of inner lamella.

planchet and then counting, contained no Ca^{45}. Thus, there was no reabsorption of Ca^{45} from the old valves prior to molting that was retained and used for calcifying the new valves. There was no uptake of Ca^{45} in either the dead control animals or in the valves discarded during the first molt in Ca^{45} and left in the containers for the duration of the experiment.

Determination of calcium storage

The ostracods used in this experiment were allowed to molt in a medium containing Ca^{45}. Therefore, the valves calcified during this molt contained Ca^{45} and had counts (400–500 cpm) equivalent to the radioactive valves recovered in the calcium reabsorption experiment above. Any significant storage of calcium in the tissues of the ostracod should be reflected in a higher cpm as the time of the next

molt approached. The ostracods were kept in the Ca^{45} medium for 24, 48, and 72 hours after molting to determine whether there would be an additional Ca^{45} buildup that could represent calcium stored for the following molt and subsequent valve calcification.

In the ten ostracods exposed to Ca^{45} for 24 hours after molting there was a range from 403 to 536 cpm with a mean of 482 cpm. In the 10 exposed for 48 hours after molting there was a range from 437 to 534 cpm with a mean of 486 cpm and in the 10 exposed for 72 hours after molting there was a range from 448 to 515 cpm with a mean of 485 cpm. The controls only registered background. With the exception of 4 counts all the numbers lie within the 95% probability level for the deviation of the mean, indicating that there is no significant variation in

TABLE II

Results of autoradiography showing time of calcification during molting process. During specimen preparation the valves were decalcified. Thus, the autoradiographs show only the Ca^{45} present in the cells active in calcification (the cells of the outer lamella) and not the increasing accumulation of Ca^{45} in the valves. At the end of seven hours the valves are calcified and the amount of Ca^{45} detectable in the cells of the outer lamella drops to zero.

Time after molting	Number of sections observed (animals)	Number of sections showing incorporation of Ca^{45}
5 minutes	120 (3)	0
30 minutes	160 (4)	160
1 hour	80 (2)	80
2 hours	120 (3)	80
3 hours	200 (5)	110
6 hours	160 (4)	90
7 hours	160 (4)	90
8 hours	80 (2)	0
11 hours	80 (2)	0
14 hours	80 (2)	0
18 hours	80 (2)	0

the observed counts. Therefore, these results indicate that there is no buildup or storage of environmental calcium within the body of the animal prior to the molt.

Histology

The general histological features of the duplicature of *Heterocypris* as determined by examination of sections stained with either haematoxylin or fast green are presented in Figure 1. The cells of the outer lamella are large, irregular in shape, and have processes extending outward to the valve and inward into the space between the outer and inner lamellae. These cells appear to be amoeboid and, during calcification, their positions within the lamella appear to change so they are found concentrated in the area being calcified. In the early stages of calcification they are concentrated at the margins of the valve, and, as these portions are calcified, they move in toward the mid-region which is the last part of the valve to be calcified.

The cells of the inner lamella are smaller in size and more regular in shape. Instead of being a loose aggregation of cells, they are organized into a more closely

packed epithelium. Although the cells have small processes extending into the interlamellar space, they do not have an amoeboid appearance. The cells have fewer nucleoli than those of the outer lamella, and they stain less intensely both with fast green used as a general protein stain and with toluidine blue.

Autoradiography of sectioned material

Calcification of the valves starts within thirty minutes after the molt, and the cellular activity associated with calcification is completed within seven hours (Table II). With the cessation of this activity there is no further change in the appearance of the valves, and calcification has evidently been completed. The autoradiographs show a localization of the isotope only in the cells of the outer lamella. They also show that calcification starts at the margins of the valves and gradually proceeds toward the mid-region where calcification is finally completed.

Mineral composition of values

The valves are mineralized exclusively with calcite.

Discussion

Several conclusions may be drawn from the results presented here. The first is that calcium dissolved in the water is the source of calcium for calcifying the valves of *Heterocypris*, although the incorporation pathways are unknown. Second, the animal does not reabsorb calcium from the old valves prior to the molt, and it does not build up a major store of calcium from other sources preparatory to molting. Third, calcification of the new valves is completed within seven hours after the molt and follows a pattern from the dorsal and ventral edges medially and from the anterior to the posterior of the animal. Finally, the cells of the outer lamellae are responsible for the deposition of calcite in the valves.

The Ca^{45} added to the culture water was incorporated into the new ostracod valves during calcification following the next molt. Whether the Ca^{45} was first incorporated into the food by action of the bacterial and fungal microfauna associated with the rice grains and was then ingested during feeding or whether it was directly absorbed by the cells of the body is not positively known. However, we favor the latter because: (1.) with no discernible method of calcium storage the animal would have to eat and digest a tremendous amount of food at the proper time to provide the large amounts of calcium necessary for the valves, (2.) in the many autoradiographs of *Heterocypris* used in this study there was no localization of Ca^{45} in the gut region or in the food it contained and (3.) the only cellular concentration of Ca^{45} found during the entire calcification process was in the cells of the outer lamella.

Van Morkhoven (1962) has reported that specimens of *Cypris pubera*, when fed on a diet of crushed snails rich in calcium, developed secondary thickening of the valves. It would appear from this that in *C. pubera* there is an assimilation of calcium from the food source. There could also have been an enrichment of the water in the culture by the dissolution of some of the crushed snail shell. Our attempts to duplicate this experiment with *Heterocypris* failed because the ostracods

would not eat crushed snails taken from their native pond nor would they eat any other kind of meat offered to them. Perhaps a difference in dietary requirements reflects a difference in calcification mechanisms.

Our finding that calcium is neither stored nor reabsorbed by *Heterocypris* is also different from Fassbinder's (1912) report that in *C. vidua* the immediate source of calcium for valve calcification is calcium stored in the body at least five days prior to molting. This was shown by culturing the ostracods for that period of time in calcium-free water with calcium-free food and finding they could still calcify their new valves after molting. Although we believe that modern tracer methods are more reliable than the methods available to Fassbinder and that this difference could account for our divergent results, there may be alternative explanations. The possibility exists that under normal conditions of adequate available environmental calcium an ostracod does not store calcium in its tissues or reabsorb calcium from the old valves prior to molting. However, under conditions of calcium stress when environmental calcium is not available, calcium may be withdrawn from the old valves and stored for calcification of the new ones.

If the calcium of the valves is not reused, and it was not in our experiments, the problem becomes one of calcium storage. In some highly ornamented benthic species the valves must weigh several times as much as the weight of the organic material of the body. Where and in what form could this amount of calcium be stored? The presence of gastroliths, such as are found in the decapod crustaceans, is doubtful, and considering the small size of the digestive tract, it is doubtful that if they were present, they could store the amount of calcium needed for valve calcification. For these reasons it is our opinion that *Heterocypris* and probably most ostracods have not evolved the calcium storage mechanisms of the decapod crustacea, but instead have evolved cellular mechanisms for the rapid concentration and deposition of this element.

We believe that the cells of the outer lamella are responsible for calcifying the valves because they are in contact with the calcified valves by means of their numerous processes, because they are concentrated in the region of active calcification, and because they are the only cells to show Ca^{45} incorporation in autoradiographs. The lack of Ca^{45} in any other cells of the body during the entire calcification process leads to the tentative conclusion that the calcium is not transported through the body of the animal but is directly concentrated from the water by the cells of the outer lamella and transported intracellularly through the cell processes to the site of deposition in the valve.

In his study of *C. vidua* Kesling found no morphological differences between the cells of the inner and outer lamella although he postulated a functional difference for the two. If he had prepared a sequence of sections of animals at various periods of the molting cycle, perhaps morphological differences would have become apparent. In *Heterocypris* sp. the differences are marked during this time, and need further study. There appears to be an increase in the number of cells of both lamellae as the time of molting approaches, and during the calcification phase a movement of cells of the outer lamella occurs as explained above. Some of the changes are related to the production of mucopolysaccharides for the new exoskeleton and probably for the elaboration of the enzymes utilized in the molt. We have stained sections taken from animals at various time intervals during the

molting cycle with the PAS reaction and have noted a marked increase in the amount of polysaccharides in the duplicature just before molting, but this needs more study before the complexities of the process are understood.

Summary

Observations on living ostracods (*Heterocypris*) show that instars one to four require one to two days between molts, and instars five to eight require two to three days between molts.

Tracer experiments with Ca^{45} show that during the molting cycle the animal neither reabsorbs Ca from the old valves prior to molt nor stores Ca preparatory to molt.

Autoradiographs indicate that calcification of the valves occurs within seven hours after the molt and proceeds from the dorsal and ventral edges towards the valve center. The autoradiographs and other stained preparations indicate that the amoeboid-like cells of the outer lamella elaborate the calcite portion of the exoskeleton.

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